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A Gene in the *Pseudomonas syringae* pv. *tomato* Hrp Pathogenicity Island Conserved Effector Locus, *hopPtoA1*, Contributes to Efficient Formation of Bacterial Colonies in Planta and Is Duplicated Elsewhere in the Genome

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The ability of *Pseudomonas syringae* to grow in planta is thought to be dependent upon the Hrp (type III secretion) system and multiple effector proteins that this system injects into plant cells. ORF5 in the conserved effector locus of the *P. syringae* pv. *tomato* DC3000 Hrp pathogenicity island was shown to encode a Hrp-secreted protein and to have a similarly secreted homolog encoded in an effector-rich pathogenicity island located elsewhere in the genome. These putative effector genes were designated *hopPtoA1* and *hopPtoA2*, respectively. DNA gel blot analysis revealed that sequences hybridizing with *hopPtoA1* were widespread among *P. syringae* pathovars, and some strains, like DC3000, appear to have two copies of the gene. *uidA* transcriptional fusions revealed that expression of *hopPtoA1* and *hopPtoA2* can be activated by the HrpL alternative sigma factor. *hopPtoA1* and *hopPtoA1/hopPtoA2* double mutants were not obviously different from wild-type *P. syringae* pv. *tomato* DC3000 in their ability to produce symptoms or to increase their total population size in host tomato and *Arabidopsis* leaves. However, confocal laser-scanning microscopy of GFP (green fluorescent protein)-labeled bacteria in *Arabidopsis* leaves 2 days after inoculation revealed that the frequency of undeveloped individual colonies was higher in the *hopPtoA1* mutant and even higher in the *hopPtoA1/hopPtoA2* double mutant. These results suggest that *hopPtoA1* and *hopPtoA2* contribute redundantly to the formation of *P. syringae* pv. *tomato* DC3000 colonies in *Arabidopsis* leaves.

The plant pathogenic bacterium *Pseudomonas syringae* is dependent on the Hrp (type III) protein secretion system to grow in the intercellular spaces of plant leaves and cause disease (Alfano and Collmer 1997). The Hrp system is thought to inject virulence (effector) proteins into plant cells, and collectively, these proteins are primary determinants of pathogenicity and host range (Kjemtrup et al. 2000). *P. syringae* is a host-specific pathogen whose strains can be assigned to more than 40 pathovars based on their specificity for different plant species and to races within pathovars based on specificity for different cultivars of the host species. Incompatible interactions with resistant plant species or cultivars are marked by the hypersensitive response (HR), a rapid, defense-associated programmed death of plant cells in contact with the pathogen. The ability of *P. syringae* to elicit the HR is also dependent on the Hrp secretion system and the (largely unknown) collection of effector proteins that it injects into plant cells.

Although *P. syringae* mutants blocked in the Hrp secretion pathway are strikingly deficient in their plant interactions, the phenotypes of mutants deficient in individual effector protein genes are typically subtle and detectable only as quantitative reductions in pathogen growth in host tissues (Kjemtrup et al. 2000). However, effector protein genes can have a dramatic phenotype when transferred into a compatible race of *P. syringae* that is then inoculated into a host cultivar that carries a resistance (*R*) gene that directs the defensive recognition of the effector (Keen 1990). Such recognition results in the HR instead of pathogenesis. Most effector genes known so far have been identified by this gain-of-function avirulence phenotype and are accordingly called *avr* genes. Unfortunately, it has not been possible to determine the full inventory of effector genes for any strain of *P. syringae* using plant reaction phenotypes, because mutant phenotypes for individual effector genes are either lacking or too difficult to detect in mutant screens and avirulence phenotypes are dependent on the unpredictable presence of cognate *R* genes in test plants.

Properties of effector genes that are independent of plant reaction assays include association with pathogenicity islands, expression by Hrp promoters (which are activated by the HrpL alternative sigma factor), and secretion in culture by the Hrp system. Genes encoding the *P. syringae* Hrp secretion system are located on a pathogenicity island (Pai) with effector genes flanking both sides of the core six operons required for regulation and formation of the translocator apparatus (Alfano et al. 2000). An exchangeable effector locus (EEL), encoding diverse putative effector proteins, is located downstream of *hrpK*. A conserved effector locus (CEL), carrying at least 10 open reading frames (ORFs) that are conserved between *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000, is located on the other side of the core *hrp/hrc* cluster, flanking the *hrpRS* operon. Deletion of the *P. syringae* pv. *tomato* DC3000 EEL slightly reduces bacterial growth in tomato, whereas deletion

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of *avrE* and the next five ORFs of the CEL abolishes pathogenicity in tomato (Alfano et al. 2000). The *P. syringae* pv. *tomato* DC3000 CEL contains a large intergenic region and seven operons, six of which are preceded by Hrp boxes (Alfano et al. 2000). The ORFs deleted in the CEL mutation encode AvrE and a putative chaperone, a second candidate effector and chaperone (ORF3 and ORF4), HrpW (a harpin), and an unknown protein (ORF5). ORF5 appears to be in a monocistronic operon preceded by a Hrp box and is the focus of this work.

The ability to travel the Hrp pathway is a general property of Hrp effector proteins that can be assayed using the *Erwinia chrysanthemi* Hrp system heterologously expressed in *Escherichia coli* (Ham et al. 1998). *E. coli*(pCPP2156) secretes in culture all *P. syringae* effectors that have been tested, including AvrB, AvrPto, AvrRpt2, and HopPsyA (Ham et al. 1998; Mudgett and Staskawicz 1999; van Dijk et al. 1999). HopPsyA has an avirulence phenotype when heterologously expressed in *P. syringae* pv. *tabaci* (Alfano et al. 1997; Collmer et al. 2000), but a cognate tobacco *R* gene has not been defined. The protein has been designated HopPsyA (for Hrp-dependent outer protein of *P. syringae* pv. *syringae*) based on its secretion phenotype, as proposed for novel Hrp-secreted effector proteins (Alfano and Collmer 1997). It is also noteworthy that the *E. coli*(pCPP2156) Hrp secretion system secretes in culture the well-studied *P. syringae* effector AvrB, even though secretion of AvrB (even under conditions shown to be optimal for the secretion of other Avr/Hop proteins) has yet to be observed with *P. syringae* (van Dijk et al. 1999). Thus, the *E. coli*(pCPP2156) Hrp secretion system is well suited for testing candidate effectors for their ability to be secreted in a Hrp-dependent manner.

The lack of any phenotype in compatible (pathogenic) interactions with host plants for many *P. syringae* effector mutants has thwarted not only detection of new effectors but also study of the function of those effectors that have already been identified by their avirulence phenotype. A priori, we would expect effectors to suppress host defenses and promote nutrient release to bacteria in the apoplast. However, little is known about the biochemical activity or virulence function of *P. syringae* effectors, other than the ability of some to enhance growth in planta, as measured by colony-forming units released from crushed leaf tissue. Similarly, the reason that mutations in individual effector genes have only minor virulence phenotypes is most likely redundancy, but this has not been investigated by identification and mutagenesis of redundant effectors or by analysis of effector mutants with cell biological assays that might detect phenotypes too subtle for bacterial population assays.

In this work, we show that the *P. syringae* pv. *tomato* DC3000 CEL ORF5 product is secreted by the *E. coli* (pCPP2156) Hrp system (and, hence, is designated HopPtoA1), that there is a similarly secreted homolog (designated HopPtoA2) that is encoded elsewhere in the genome, that *hopPtoA1* and *hopPtoA2* are expressed in a HrpL-dependent manner, and that *hopPtoA1*/*hopPtoA2* mutants have a novel phenotype in host leaves that is observable by confocal laser-scanning microscopy of bacterial colonies labeled with *Aequorea victoria* green fluorescent protein (GFP).

RESULTS

hopPtoA1 is located in the Hrp Pai CEL, divergently oriented between ORF6 and *hrpW*.

The *P. syringae* pv. *tomato* DC3000 Hrp Pai CEL was isolated on two overlapping cosmids, pCPP2357 and pCPP3016 (Alfano et al. 2000). Seven putative operons, six of which are preceded by Hrp boxes, and one large intergenic region were identified (Fig. 1A). The operon encoding the 1,461-bp ORF5 now designated *hopPtoA1* is a single-gene operon preceded by Hrp box promoter sequence GGAACC-N16-CCACACA.

Sequences hybridizing with *hopPtoA1* are found in other phytopathogenic bacteria and elsewhere in the *P. syringae* pv. *tomato* DC3000 genome.

We examined the distribution of *hopPtoA1* in *P. syringae* pathovars by DNA gel blot hybridization analysis, using a polymerase chain reaction (PCR)-amplified *P. syringae* pv. *tomato* DC3000 *hopPtoA1* fragment as a probe. The *hopPtoA1* probe hybridized to at least one distinct band for *P. syringae* pv. *syringae* B728a and 61 (weak), pv. *angulata* 9, *glycinea* races 1, 4, and 6, *lachrymans* 859, *phaseolicola* 343, and *tabaci* ATCC 11528. Very weak hybridization was observed with *P. syringae* pvs. *papulans* and *pisi* (Fig. 2A).

In this hybridization analysis, we noted that additional and unexpected DNA fragments were hybridizing with the *hopPtoA1* probe, suggesting the presence of a *hopPtoA1* homolog elsewhere in the *P. syringae* pv. *tomato* DC3000 genome. In order to confirm this possibility, total DNA from the *P. syringae* pv. *tomato* ΔCEL mutant strain CUCPB5115 (Alfano et al. 2000), in which most of the CEL locus including *hopPtoA1*, is replaced with a ΩSp/Sm^r interposon, was digested with *Eco*RI and was probed with the PCR-amplified *hopPtoA1*. The probe hybridized distinctly and strongly to an approximately 7.5-kb band (Fig. 2B), suggesting that *P. syringae* pv. *tomato* DC3000 carries a *hopPtoA1* homolog elsewhere in the genome (Fig. 1).

hopPtoA2 is part of an apparent pathogenicity island that is not linked with the Hrp Pai.

The *P. syringae* pv. *tomato* ΔCEL mutant CUCPB5115 (Alfano et al. 2000) was digested with *Hin*dIII, and the 11.9-kb fragment hybridizing with a PCR-amplified *hopPtoA1* probe was cloned into pBluescriptII SK and was sequenced. A 1,464-bp ORF sharing 81% identity to *hopPtoA1* was found in an apparent monocystronic operon preceded by the Hrp box promoter sequence GGAACC-N16-CTACACA and was designated *hopPtoA2* (Figs. 1B and 3A). Another ORF cloned in this fragment is preceded by a Hrp box, the 1,404-bp *avrPphD2*$_{Pto}$. The first 500 bp of this ORF show similarity (81% identity) to the first 500 bp of the *P. syringae* pv. *phaseo-*

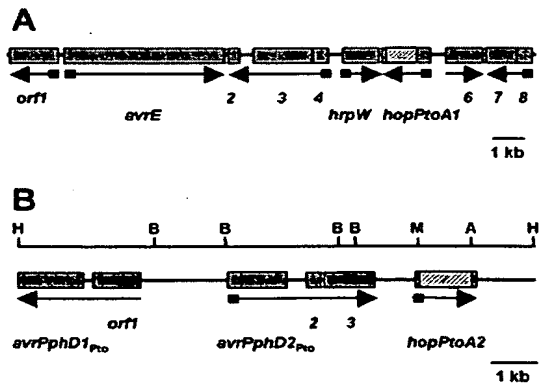

Fig. 1. Physical maps of the location of *hopPtoA1* and *hopPtoA2* and mutations. A, Genetic organization of the CEL locus. B, Restriction and physical maps of the cloned fragment carrying *hopPtoA2*. Shaded boxes indicate genes; diagonally lined areas denote regions deleted in *hopPtoA1* and *hopPtoA2*. Arrows indicate the direction of transcription; small black boxes denote the presence of a Hrp box. H = *Hin*dIII; B = *Bam*HI; M = *Mlu*I; and A = *Age*I.

licola race 4 avirulence gene *avrPphD* (Arnold et al. 2001), with no detectable similarity for the rest of the coding sequence. The full length of the predicted amino acid sequence shows 98% identity to AvrPphD, 77% identity to the *Xanthomonas campestris* pv. *vesicatoria* Hrp-secreted protein XopB (Noël et al. 2001), and 34% identity to a *Ralstonia solanacearum* AvrPphD-related protein (Salanoubat et al. 2002). Further upstream, beyond a long intergenic region, the nucleotide sequence of the 801-bp ORF1 shows greater than 92% identity to several transposaselike genes and insertion sequences found in *Pseudomonas* spp. (Chablain et al. 2001; Habe et al. 1996; Yamada et al. 1986).

The nucleotide sequence of the next truncated ORF upstream of ORF1, *avrPphD1*$_{Pto}$, has 90% identity to the *P. syringae* pv. *phaseolicola avrPphD* (Arnold et al. 2001) and 79% identity to the *X. campestris* pv. *vesicatoria xopB* (Noel et al. 2001). BLASTN and BLASTP searches with ORF2 and ORF3 sequences reveal no homologs in the databases, although it is noteworthy that the operon structure, a long ORF preceded by a much shorter one, commonly observed for chaperone/effector pairs, resembles those found for ORF4/ORF3 and ORF8/ORF7 in the CEL locus of the *P. syringae* pv. *tomato* DC3000 Hrp Pai (Fig. 1A). The average G + C content (55.2%) of the ORFs in this region is significantly lower than the average of 59% of *P. syringae* (Palleroni 1984). Overall, the linkage of *hopPtoA2* with several virulence-associated genes with a low G + C content, some preceded by Hrp boxes, and also with sequences related to mobile genetic elements suggest that it is part of an apparent pathogenicity island.

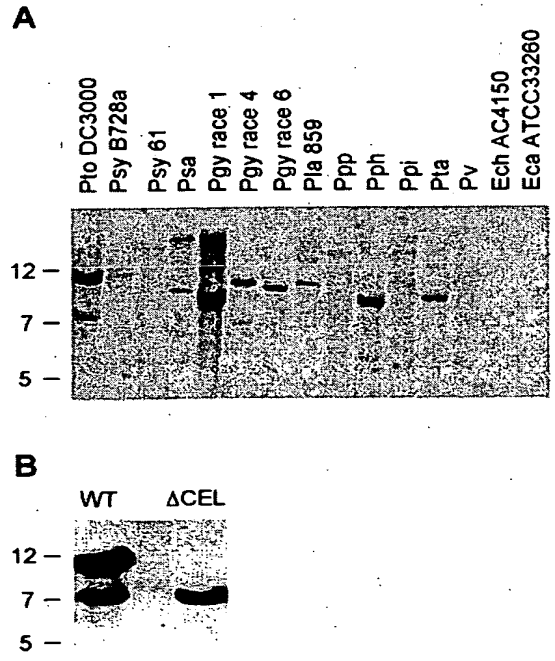

Fig. 2. DNA gel blot analysis of the hybridization of *hopPtoA1* to total DNA from other bacterial pathogens and to the *Pseudomonas syringae* pv. *tomato* ΔCEL mutant. **A**, DNA from indicated pathogens was isolated, digested with *Eco*RI, resolved on a 0.5% agarose gel, transferred to an Immobilon-N membrane, and hybridized with a ^{32}P-labeled *hopPtoA1* probe. Pto = *P. syringae* pv. *tomato*; Psy = *P. Pseudomonas syringae* pv. *syringae*; Psa = *P. syringae* pv. *angulata*; Pgy = *P. syringae* pv. *glycinea*; Pla = *P. syringae* pv. *lacrymans*; Ppp = *P. syringae* pv. *papulans*; Pph = *P. syringae* pv. *phaseolicola*; Ppi = *P. syringae* pv. *pisi*; Pta = *P. syringae* pv. *tabaci*; Pv = *P. viridiflava*; Ech = *Erwinia chrysanthemi*; and Eca = *Erwinia carotovora* subsp. *atroseptica*. **B**, DNA from the *P. syringae* pv. *tomato* ΔCEL mutant CUCPB5115 was subjected to DNA blot analysis as indicated above. WT = *P. syringae* pv. *tomato* DC3000; and ΔCEL = *P. syringae* pv. *tomato* CUCPB5115.

The HopPtoA1 and HopPtoA2 proteins are highly similar.

hopPtoA1 is predicted to encode a basic, alanine-rich (83 alanine residues, 17.1%) 486-residue protein of 50.7 kDa with a pI of 9.06. HopPtoA1 is like harpins in lacking cysteines (Fig. 3B) (Alfano and Collmer 1996; Alfano et al. 2000; He et al. 1993; Charkowski et al. 1998; Wei et al. 1992). However, unlike harpins, HopPtoA1 is not rich in glycine (35 glycine residues, 7.2%) and has a relatively high pI. *hopPtoA2* encodes a protein with 77% identity to HopPtoA1. HopPtoA2 is predicted to be a 487-residue, basic, alanine-rich (73 alanine residues, 15.0%) protein of 51.1 kDa with a pI of 9.16. Like HopPtoA1, it has 35 glycine residues (7.2%) but, unlike HopPtoA1, it has a cysteine residue at position 39 (Fig. 3B), which further suggests that HopPtoA proteins are not harpins. BLASTP searches with the predicted protein sequences of HopPtoA1 and HopPtoA2 of the GenBank database and of 86 microbial genome sequences currently deposited at NCBI reveal that they share 32 and 35% identity, respectively, to a putative transmembrane protein of *Ralstonia solanacearum* (Salanoubat et al. 2002). No discernible structural motifs were found in the HopPtoA1 and HopPtoA2 sequences using the tools InterProScan, Pfam, 3D-pssm, and Loopp.

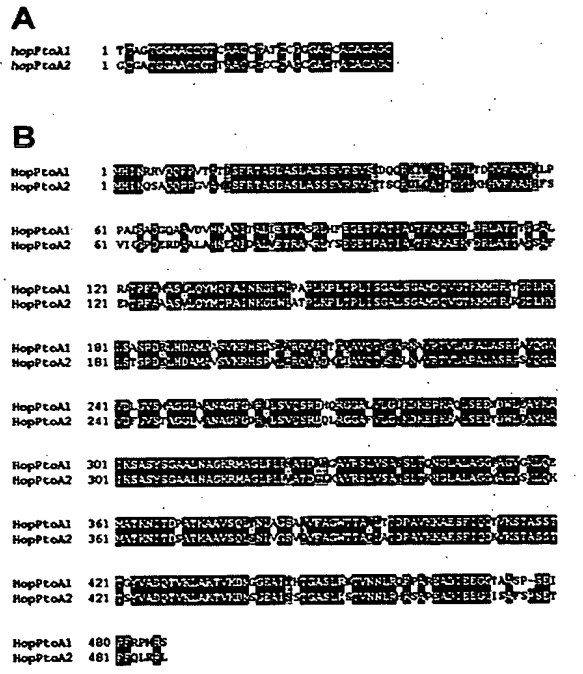

Fig. 3. Alignment of the nucleotide sequences of the *hopPtoA1* and *hopPtoA2* Hrp boxes and the amino acid sequences of the HopPtoA1 and HopPtoA2 proteins. **A**, Black boxes indicate conserved nucleotides in *hopPtoA1* and *hopPtoA2* Hrp boxes and flanking nucleotides. **B**, In this alignment of the HopPtoA1 and HopPtoA2 predicted protein sequences, black boxes indicate identical residues, gray boxes denote conserved residues, and a white background indicates different residues. Alignments were obtained with ClustalW and BoxShade.

uidA transcriptional fusions reveal that _hopPtoA1_ and _hopPtoA2_ can be expressed in a HrpL-dependent manner.

The presence of Hrp box sequences in the promoter regions of *hopPtoA1* and *hopPtoA2* prompted us to test whether these genes are HrpL-inducible. We constructed single-copy, transcriptional fusions with *uidA* in the *P. syringae* pv. *tomato* DC3000 *hrp/hrc* mutant strain CUCPB5114 and expressed *hrpL* constitutively under the control of the *nptII* promoter in plasmid pCPP5032. pCPP5031, a plasmid carrying only the *nptII* promoter, was used as a HrpL$^-$ control (Fouts et al. 2002). We cultured the resulting strains, CUCPB5135(pCPP5031), CUCPB5135(pCPP5032), CUCPB5136(pCPP5031), and CUCPB5136(pCPP5032), in liquid AB-citrate medium (Chilton et al. 1974) and measured GUS activity. The GUS activity of CUCPB5135, carrying a *hopPtoA1::uidA* fusion, was 27-fold higher in the presence of pCPP5032 than in the presence of pCPP5031. The GUS activity of CUCPB5136, carrying a *hopPtoA2::uidA* fusion, was 16-fold higher in the presence of pCPP5032 (Fig. 4). The fact that the expression of *hopPtoA1* and *hopPtoA2* is strongly induced in the presence of HrpL demonstrates that both genes have functional Hrp promoters.

HopPtoA1 and HopPtoA2 are secreted in a Hrp-dependent manner.

Because effector proteins travel the type III secretion pathway, we decided to check whether the proteins encoded by the *hopPtoA* genes are secreted by a Hrp secretion system. We constructed C-terminal fusions of *hopPtoA1* and *hopPtoA2* with the FLAG epitope in pCPP3020 and pCPP5049, respectively, and introduced these plasmids into *E. coli* cells carrying the *Erwinia chrysanthemi* Hrp cluster in cosmid pCPP2156 and its mutant derivative pCPP2368. We then determined the distribution of flagged proteins in the supernatant and cell-bound fractions. Both HopPtoA1 and HopPtoA2 were detected in the supernatant fraction of *E. coli* cells carrying pCPP2156, but not in the supernatant fraction of *E. coli* cells carrying the Hrp$^-$ derivative pCPP2368 (Fig. 5). In order to rule out the possibility that the presence of the HopPtoA proteins in the supernatant fractions was due to cell lysis, we performed immunodetection using anti-β-galactosidase antibodies. The β-galactosidase protein was detected only in the cell-bound fraction, regardless of whether the *E. coli* cells carried pCPP2156 or pCPP2368 (Fig. 5). These results demonstrate that both HopPtoA1 and HopPtoA2 are secreted in a Hrp-dependent manner.

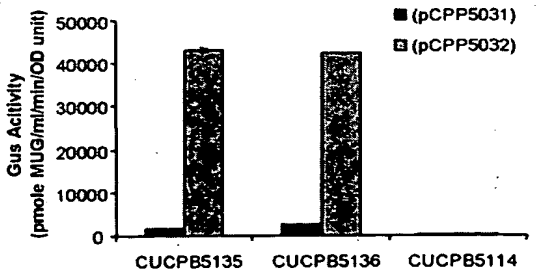

Fig. 4. Expression of *hopPtoA1* and *hopPtoA2* promoters in the presence of HrpL. *Pseudomonas syringae* pv. *tomato* strains CUCPB5135 (Δ*hrp/hrc*::ΩCm^r *hopPtoA1*::*uidA*), CUCPB5136 (Δ*hrp/hrc*::ΩCm^r *hopPtoA2*::*uidA*), and CUCPB5114 (Δ*hrp/hrc*::ΩCm^r) carrying pCPP5031 (vector control; black bars) or pCPP5032 (HrpL$^+$; shaded bars) were grown at 30°C for 6 h in liquid AB-citrate and GUS activity determined. Vertical lines indicate standard errors.

***hopPtoA1* and *hopPtoA2* single and double mutants are not obviously reduced in their ability to grow in planta or to produce disease symptoms.**

Marker-exchange mutagenesis was used to construct *P. syringae* pv. *tomato* mutants CUCPB5106 (Δ*hopPtoA1*::*nptII*), CUCPB5133 (Δ*hopPtoA2*::*aadA1*), and CUCPB5134 (Δ*hopPtoA1*::*nptII* Δ*hopPtoA2*::*aadA1*). Mutant constructions were confirmed with DNA gel blot analysis (data not shown). Tobacco leaves were infiltrated with wild-type *P. syringae* pv. *tomato* DC3000, CUCPB5106, CUCPB5133, and CUCPB5134 at three concentrations: optical density at 600 nm (OD_{600}) of 0.8 and 0.2 and a 1:10,000 dilution of the latter concentration. Leaves were examined 24 h later to determine the percentage of infiltrated tissue that was necrotic. No difference was observed between the wild type and any of the *hopPtoA* mutants (data not shown). The *hopPtoA1* gene did not inhibit the HR elicited by *P. fluorescens*(pHIR11), nor did it complement the HR-deficient phenotypes of pHIR11 *hrpZ* or *hopPsyA* mutants (data not shown). Thus, HopPtoA1 does not appear to be an analog of the HrpZ harpin or HopPsyA.

To determine if the *hopPtoA* mutants were reduced in virulence, tomato leaves inoculated with *P. syringae* pv. *tomato* DC3000, CUCPB5106, CUCPB5106(pCPP2475), CUCPB5133, CUCPB5133(pCPP5047), CUCPB5134, and CUCPB5134(pCPP2475, pCPP5047) were monitored over a period of 4 days. The *hopPtoA* mutants did not cause reduced symptoms in tomato leaves (Fig. 6A through D) and did not show reduction in bacterial multiplication compared with the wild type (Fig. 6E). Overexpression of *hopPtoA* genes in plasmids pCPP2475 and pCPP5047 did not result in changes in the ability of wild-type *P. syringae* pv. *tomato* DC3000 to grow in planta or to produce disease symptoms. Similar results were observed when *Arabidopsis* leaves were inoculated with the same strains (data not shown).

Confocal microscopy with GFP-labeled bacteria reveals that *hopPtoA1* and *hopPtoA2* double mutants are significantly reduced in their ability to develop colonies *in planta*.

We transformed *P. syringae* pv. *tomato* DC3000 wild type and *hopPtoA* mutant strains CUCPB5106, CUCPB5133, and CUCPB5134 with pTB93F or pGFP-TIR and examined the sizes of the resultant GFP-labeled colonies that formed in

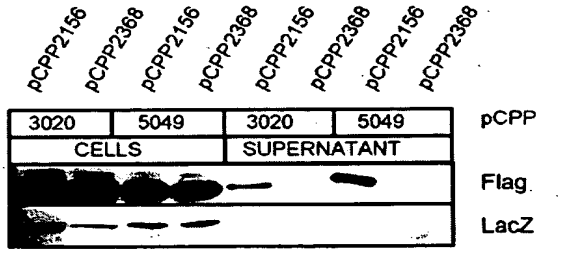

Fig. 5. Immunoblot analysis of the secretion of FLAG-epitope-tagged HopPtoA1 and HopPtoA2 by *E. coli* carrying the *Erwinia chrysanthemi hrp/hrc* cluster. Bacteria were grown in liquid LM at 30°C to an OD_{600} of 0.4 and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 500 μM. Cultures were grown further to an OD_{600} of 0.8 and separated into cell-bound and supernatant fractions by centrifugation. Proteins were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and immunostained with antibodies against the FLAG epitope and β-galactosidase. pCPP2156 carries the *Erwinia chrysanthemi hrp/hrc* cluster (Hrp$^+$), pCPP2368 is a pCPP2156 mutant derivative (Hrp$^-$), pCPP3020 expresses *hopPtoA1*, and pCPP5049 expresses *hopPtoA2* under the control of the *tac* promoter.

planta. In three separate experiments, we infiltrated *Arabidopsis* leaves with wild type and mutant strains and then observed the size of individual colonies in planta 2 days post infiltration using confocal laser-scanning microscopy. The *hrcC* mutant CUCPB5116 carrying pTB93F was also observed in *Arabidopsis* leaves. *P. syringae hrcC* mutants are blocked in type III secretion, have strong Hrp$^-$ phenotypes, and are unable to grow in planta (Alfano and Collmer 1997). The largest GFP-labeled colony or cell aggregate formed by the *hrcC* mutant was approximately 250 μm^3, whereas the wild type produced colonies with a range of sizes and the majority of these were much larger than 250 μm^3 (data not shown). We accordingly used 250 μm^3 as the threshold size for assessing the frequency of viable parasitic colonies in planta. The *hopPtoA1* mutant CUCPB5106 was also visible in a wide range of colony sizes, showing a higher frequency of colonies less than 250 μm^3 than in the wild type (Fig. 7B, Table 1). Although the frequency of colonies less than 250 μm^3 was not significantly higher than in the wild type in this experiment, it was consistently higher in several experiments, and complementation of the *hopPtoA1* mutation with pCPP2475 restored the wild-type frequency in all cases (data not shown). Significantly more colonies with less than 250 μm^3 were observed with *hopPtoA1/hopPtoA2* double mutant CUCPB5134 ($p < 0.001$) (Table 1). Thus, CUCPB5134 appears significantly reduced in its ability to develop parasitic colonies in planta.

DISCUSSION

We have found the *P. syringae* pv. *tomato* DC3000 CEL ORF5 to encode a Hrp-secreted protein and to have a similarly secreted homolog in an effector-rich pathogenicity island that is not linked to the Hrp pathogenicity island CEL region. Expression of *hopPtoA1* and *hopPtoA2* is activated by HrpL, and mutants have a phenotype observable by confocal laser-scanning microscopy that suggests a role for these proteins in colony development during pathogenesis. Several aspects of HopPtoA redundancy, secretion, expression, and contribution to virulence warrant further discussion.

Our previous observation that CEL ORF5 sequences are conserved in the distantly related *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* (Alfano et al. 2000) is consistent with the present DNA gel blot data suggesting that *hopPtoA* is widespread among *P. syringae* pathovars. Similarly, our recent genomewide analysis of ORFs downstream of Hrp box sequences in a genomic draft sequence of *P. syringae* pv. *tomato* DC3000 independently indicated the presence of a HopPtoA homolog (Fouts et al. 2002). We show here that the high similarity between *hopPtoA2* and *hopPtoA1* extends throughout the proteins and that multiple *hopPtoA* sequences are present in some, but not all, other *P. syringae* pathovars.

Interestingly, *hopPtoA2* in DC3000 is linked to two *P. syringae* pv. *phaseolicola avrPphD* homologs (Arnold et al. 2001) and to sequences related to insertion elements. The average G + C content (55.2%) of the ORFs in this region is significantly lower than the average of *P. syringae* (59%) (Palleroni 1984), and one of these ORFs, *avrPphD2*$_{Pto}$, is also preceded by a Hrp box. *avrPphD* was originally isolated from *P. syringae* pv. *phaseolicola* race 4 through its ability to confer an avirulence phenotype to *P. syringae* pv. *pisi* on the normally susceptible pea cultivar Kelvedon Wonder (Wood et al. 1994). *avrPphD* is also widespread among *P. syringae* pathovars, but its mutation in *P. syringae* pv. *phaseolicola* race 7 does not result in reduction of bacterial multiplication or in virulence towards bean (Arnold et al. 2001). Noël and associates (2001), using cDNA-AFLP (amplified fragment length polymorphism) analysis, recently identified an *avrPphD* homolog in *X. campestris* pv. *vesicatoria*, *xopB*, which is also conserved among four *X. campestris* pv. *vesicatoria* strains tested. Mutation of *xopB* in *X. campestris* pv. *vesicatoria* 85-10 does not affect pathogenicity or bacterial multiplication in susceptible pepper Early Cal Wonder plants. Intriguingly, *xopB* is preceded by a Hrp box, which is characteristic of the Hrp regulons of *P. syringae* but not of *Xanthomonas* spp. Better knowledge of the conservation, redundancy, and genomic location of effector protein genes will, hopefully, provide clues to effector function. Our findings here suggest that *hopPtoA* may be part of both stable and mobile parts of the genome and that *hopPtoA2* was acquired by *P. syringae* pv. *tomato* DC3000 more recently.

In *P. syringae*, the Hrp secretion machinery genes and all known effector genes are preceded by Hrp box promoters and are part of the Hrp regulon (Fouts et al. 2002). This regulon is activated by a regulatory cascade that includes the σ^{54} enhancer-binding proteins HrpR and HrpS (Grimm et al. 1995; Hutcheson et al. 2001; Xiao et al. 1994) and the alternative sigma factor HrpL, which presumably interacts with Hrp boxes and activates *hrp/hrc* and effector genes (Innes et al. 1993; Shen and Keen 1993; Xiao and Hutcheson 1994; Xiao et al. 1994). We show that *hopPtoA1* and *hopPtoA2* are activated by HrpL to similar levels. This result was surprising for several reasons. The *hopPtoA2 hrp* box sequence differs from the canonical sequence and from the *hopPtoA1 hrp* box sequence in having a T in the −10 motif CTACACA, and attempts to detect HrpL-dependent induction of *hopPtoA2* using genomewide

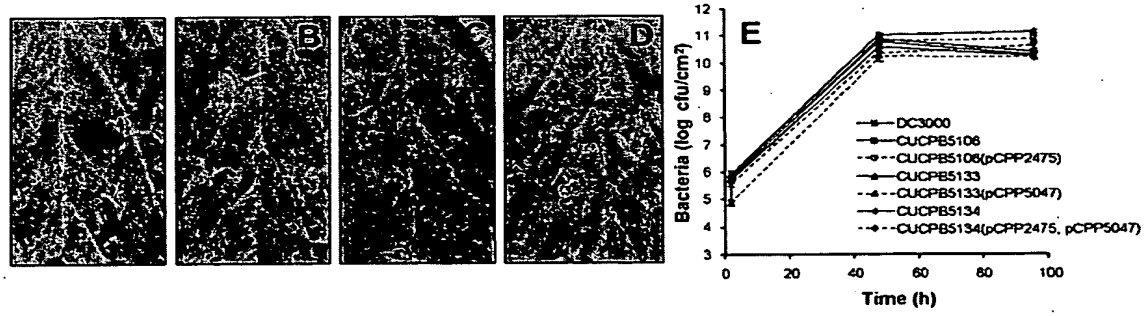

Fig. 6. Plant interaction phenotypes of *Pseudomonas syringae* pv. *tomato* mutants carrying deletions in *hopPtoA1* (CUCPB5106), *hopPtoA2* (CUCPB5133), and *hopPtoA1/hopPtoA2* (CUCPB5134). Disease symptoms of tomato leaves 4 days after inoculation (10^4 CFU/ml) with **A**, *P. syringae* pv. tomato DC3000 wild type, **B**, CUCPB5106, **C**, CUCPB5133, and **D**, CUCPB5134. **E**, Growth in tomato of *P. syringae* pv. *tomato* DC3000, CUCPB5106, CUCPB5106(pCPP2475), CUCPB5133(pCPP5047), CUCPB5134, and CUCPB5134(pCPP2475, pCPP5047). Symbols represent the mean for three replications and vertical lines indicate standard errors.

miniTn*5gus* reporter mutagenesis, microarray analysis, and Northern hybridization failed (Fouts et al. 2002). Possible explanations for this discrepancy are that *hopPtoA2* was missed by reporter transposon mutagenesis, which did not saturate the genome, and that the *hopPtoA2-uidA* transcript may be more stable than the native *hopPtoA2* mRNA that was assayed in the previous work.

The *P. syringae* Hrp system also secretes proteins that are likely to be translocation factors rather than effectors per se. Examples of these proteins are HrpA, which is the major component of the Hrp pilus (Roine et al. 1997), and the harpins HrpZ (He et al. 1993; Preston et al. 1995) and HrpW (Charkowski et al. 1998), which appear to be targeted to the plant cell wall (Hoyos et al. 1996) but also have effects on protoplasts and membranes (Lee et al. 2001). We observed that inoculation of tobacco leaves with the *hopPtoA1* (CUCPB5106), *hopPtoA2* (CUCPB5233), and *hopPtoA1/hopPtoA2* (CUCPB5134) mutant strains results in tissue collapse that is indistinguishable from that caused by the wild-type *P. syringae* pv. *tomato* DC3000. The fact that the *hopPtoA* mutants are able to elicit the HR when infiltrated into tobacco leaves indicates that the translocation process is not impaired and argues against *hopPtoA1* and *hopPtoA2* encoding components of the secretion apparatus.

Harpins, such as the *Erwinia amylovora* HrpN and HrpW (Kim and Beer 1998; Wei et al. 1992;) and *P. syringae* HrpZ (He et al. 1993; Preston et al. 1995) and HrpW (Charkowski et al. 1998) proteins, appear to be redundant factors that are secreted by the Hrp system and that may promote effector translocation. HopPtoA1 is like harpins in lacking cysteine residues; however, several lines of evidence argue against HopPtoA proteins being harpins. First, HopPtoA2 has a cysteine residue at position 39. Second, HopPtoA proteins are not rich in glycine residues, and they have a basic, rather than acidic, pI. Third, *hopPtoA1* expressed in trans does not complement the HR minus phenotype of a *P. fluorescence 55*(pHIR11) *hrpZ* nonpolar deletion mutant nor does it interfere with elicitation of HR in tobacco leaves by *P. fluorescence 55*(pHIR11).

Therefore, the HopPtoA proteins probably function as effectors. However, as with many effectors, standard assays failed to reveal any significant reduction in virulence or bacterial multiplication in planta of *hopPtoA1*, *hopPtoA2*, or *hopPtoA1/hopPtoA2* mutants. We inoculated tomato and *Arabidopsis* plants by infiltration using a blunt syringe and by dipping in inoculum of concentrations ranging from 10^2 to 10^5 CFU per ml. To date, evidence of the contribution to virulence of *P. syringae* pv. *tomato* DC3000 effectors using such methods has been obtained indirectly from mutations in gene homologs present in less virulent *P. syringae* pv. *tomato* strains, e.g., PT23 (Lorang et al. 1994), or from less virulent strains, e.g., T1, heterologously expressing the effector gene (Shan et al. 2000). Moreover, increased virulence of *P. syringae* pv. *tomato* DC3000 expressing heterologous effector genes has been observed only when challenging plants with enhanced disease resistance, e.g., *Arabidopsis* No-0 or *coi1* mutant, or through the use of transgenic plants expressing the effector gene (Chen et al. 2000). The reason that mutations in individual effector genes have little or no virulence phenotype is most likely redundancy, but this has not been experimentally tested.

Our DNA gel blot and genomic analyses both indicate that *P. syringae* pv. *tomato* DC3000 contains only two *hopPtoA* homologs. We mutated both of these but did not detect any phenotype, using standard assays for symptom production and total bacterial growth in planta. Consequently, we tried a cell biological assay capable of detecting subtle changes in the parasitic behavior of the mutants. Specifically, we inoculated *Arabidopsis* leaves with GFP-labeled wild-type and mutant strains and observed the development of unperturbed individual colonies. We found that, relative to the wild type, the frequency of undeveloped colonies was higher in the *hopPtoA1* mutant and even higher in the *hopPtoA1/hopPtoA2* double mutant. These results suggest that *hopPtoA1* and *hopPtoA2* contribute redundantly to the formation of *P. syringae* pv. *tomato* DC3000 colonies in *Arabidopsis* leaves, and they validate confocal laser-scanning microscopy analysis of GFP-labeled bacterial colonies as a sensitive and informative assay for effector mutant performance in planta.

Why many *hopPtoA1/hopPtoA2* mutant cells in a leaf still parasitically succeed and produce colonies as large as the wild type is unclear. Perhaps *P. syringae* pv. *tomato* DC3000 produces other effectors that have a different structure but a similar function. Alternatively, infection sites in a leaf may be heterogeneous, with only a fraction requiring the HopPtoA proteins for colony formation. To resolve this issue, we need to better understand the inventory of effector proteins in *P. syringae* pv. *tomato* DC3000 and the interactions of individual bacterial and plant cells. This work represents a first step in exploring the function of the HopPtoA proteins and in using cell biological tools to define stages in *P. syringae* pathogenesis to which individual effectors (or effector families) may be assigned.

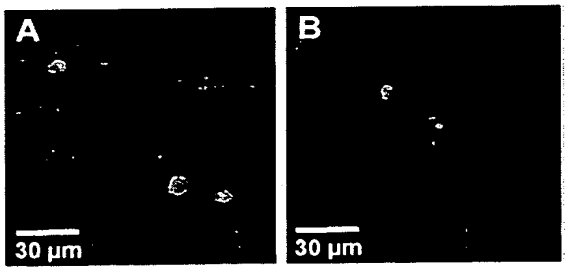

Fig. 7. Confocal laser-scanning microscopy analysis of the development of green fluorescent protein (GFP)-labeled *Pseudomonas syringae* pv. *tomato* and *hopPtoA* mutants in *Arabidopsis* leaves. Leaves were infiltrated with 10^6 CFU/ml suspensions of bacteria carrying pTB83F and were observed after 40 h by confocal laser-scanning microscopy. Light from two photomultiplier tubes was collected to detect either red light from chloroplast autofluorescence or green light from GFP. A, GFP-labeled *P. syringae* pv. *tomato* DC3000; B, GFP-labeled *hopPtoA1* mutant CUCPB5106.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains, cosmid clones, and plasmids used in this study are described in Table 2. *E. coli* cells were grown in LM (Hanahan 1985) or terrific broth (Sambrook et al. 1989) at 37°C,

Table 1. Colony sizes of green fluorescent protein-labeled *Pseudomonas syringae* pv. tomato DC3000 wild type and *hopPtoA* mutant strains in *Arabidopsis*[a]

Strain	Colony size (µm³)		
	< 250	≥ 250	Total
CUCPB5106 (*hopPtoA1*)	209 (29.2)[b]	506 (70.8)	715
CUCPB5134 (*hopPtoA1/hopPtoA2*)	290[c] (43.0)	385 (57.0)	675
Wild type	247 (25.8)	711 (74.2)	958

[a] Table probability $p < 0.0001$ (chi-square statistics).
[b] Values in parentheses indicate row percentages.
[c] Statistically different from wild type ($p < 0.001$, chi-square statistics).

and *P. syringae* cells were grown in King's B (King et al. 1954) or in *hrp*-derepressing fructose minimal medium (Huynh et al. 1989) at 30°C. Except where noted, *E. coli* DH5 and *E. coli* DH5α(Life Technologies, Grand Island, NY, U.S.A.) were used as hosts for DNA clones and the pBluescriptII SK plasmid (Stratagene, La Jolla, CA, U.S.A.) was used as a vector. The nucleotide sequence of the CEL of the *P. syringae tomato* DC3000 *hrp/hrc* cluster was determined using pv. subclones of pCPP2357 and pCPP3016. These cosmids were isolated as described by Alfano and associates (2000). GFP was constitutively expressed in plasmids pGFP-TIR (Miller and Lindow 1997) or pTB93F (Gage et al. 1996). Antibiotics were used at the following concentrations (μg/ml): ampicillin, 100; kanamycin 50; rifampin, 50; gentamycin, 10; tetracycline, 10; spectinomycin, 50.

Recombinant DNA techniques.

DNA manipulations and PCR were performed according to standard protocols (Sambrook et al. 1989; Innis et al. 1990). Oligonucleotide primers for sequencing or PCR were purchased from Integrated DNA Technology (Coralville, IA, U.S.A.) and are described in Table 3. PCR was performed with Vent or Deep Vent polymerases (New England Biolabs, Beverly, MA, U.S.A.). All DNA sequencing was done at the Cornell Biotechnology Center with an automated DNA sequencer, model 373A (Applied Biosystems, Foster City, CA, U.S.A.). DNA sequences were analyzed with the DNASTAR (Madison, WI, U.S.A.) software package. Database searches were performed using gapped BLASTN, BLASTP, and BLASTX (Altschul et al. 1997).

Table 2. Strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Strains		
Escherichia coli DH5α	*SupE44 ΔlacU169* (f80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nx^r	Life Technologies
Pseudomonas syringae pv. tomato		
DC3000	Wild type, Rif^r	Cuppels 1986
CUCPB5106	*ΔhopPtoA1::nptII*, Rif^r Km^r	This study
CUCPB5114	*Δhrp/hrc*::ΩCm^r, Rif^r Cm^r	Fouts et al. 2002
CUCPB5115	*ΔCEL*::ΩSp/Sm^r, Rif^r Sp^r	Alfano et al. 2000
CUCPB5116	*hrcC*::Tn*5Cm*, Rif^r Cm^r	Yuan and He 1996
CUCPB5133	*ΔhopPtoA2::aadA1*, Rif^r Sp^r	This study
CUCPB5134	*ΔhopPtoA1::nptII ΔhopPtoA2::aadA1*, Rif^r Km^r Sp^r	This study
CUCPB5135	*ΔhopPtoA1::uidA1::nptII*, Rif^r Km^r derivative of CUCPB5114	This study
CUCPB5136	*ΔhopPtoA2::uidA1::nptII*, Rif^r Km^r derivative of CUCPB5114	This study
P. fluorescence 55	Wild type, Nx^r	Huang et al. 1988
Plasmids		
pBluescriptII SK or KS	ColE1 mcs-*lacZ*, Ap^r	Stratagene
pRK415	Broad-host-range vector unstable in the absence of selection, Tc^r	Keen et al. 1988
pCPP45	Broad-host-range, pCPP30 with RP4 *par* region in *Stu* I site, Tc^r	D. W. Bauer (Cornell University)
pUCP24	*Escherichia-Pseudomonas* shuttle vector with *aacC1* and *lazα*, Gm^r	Olsen et al. 1982
pFLAG-CTC	For construction of C-terminal fusions to the FLAG epitope, Ap^r	Kodak
pCPP54	pCPP33 derivative carrying *SacB* and *SacR* from pUM24, Tc^r	D. W. Bauer (Cornell University)
pJQ200SK	Suicide vector with *lacZα* carrying *sacB*, Gm^r	Quandt and Hynes 1993
pHP45ΩSp/Sm	pHP45 carrying ΩSp/Sm^r for interposon mutagenesis, Ap^r Sp^r	Prentki and Krisch 1984
pGFP-TIR	pGreen carrying TIR, Gm^r	Miller and Lindow 1997
pTB93F	p*trp*-GFP-S65T in pMB393, Sp^r	Gage et al. 1996
pCPP2277	pBluescriptII SK carrying a *uidA1::nptII* cassette	D. W. Bauer (Cornell University)
pCPP2988	pBluescript II SK(–) carrying 1.5 *Hin*dIII-*Sac*I fragment from pRZ102 with *nptII* lacking terminator, Ap^r Km^r	Alfano et al. 1996
pHIR11	pLAFR3 derivative carrying 25 kb of *P. syringae 61 hrp/hrc* cluster, Tc^r	Huang et al. 1988
pCPP2156	pCPP19 derivative carrying *Erwinia chrysanthemi hrp/hrc* cluster, Sp^r	Ham et al. 1998
pCPP2368	pCPP2156::*Tn5*Cm that has HR$^-$ phenotype, Sp^r Cm^r	Ham et al. 1998
pCPP2357	Cosmid clone, pCPP47 carrying from *hrcJ* to *hrpW* of the *P. syringae* pv. tomato DC3000 Hrp pai, Tc^r.	Alfano et al. 2000
pCPP3016	Cosmid clone, pCPP47 carrying 5′ end of *avrE* to CEL edge of the *P. syringae pv.* tomato DC3000 Hrp pai, Tc^r	Alfano et al. 2000
pCPP2435	pBluescript II SK(+) carrying 11-kb *Eco*RI fragment from pCPP3016; contains *avrE*, *hrpW*, *hopPtoA1*, Ap^r	Alfano et al. 1996
pCPP2437	pBluescript II SK carrying 4.2 *Mfe*I fragment from pCPP3016, Ap^r	This study
pCPP2476	pCPP2437 *ΔhopPtoA1::nptII*, Ap^r Km^r	This study
pCPP2455	pRK415 carrying *Bam*HI fragment from pCPP2476, Tc^r Km^r.	This study
pCPP2475	pUCP24 carrying 3.0-kb *Sph*I fragment from pCPP2435, Gm^r	This study
pCPP3020	pFLA-CTC carrying *hopPtoA1*, Ap^r	This study
pCPP3021	pBluescriptII SK carrying *hopPtoA1*, Ap^r	This study
pCPP5031	pRK415 carrying *PnptII* , Tc^r	Fouts et al. 2002
pCPP5032	pRK415 carrying *P. syringae pv.* tomato DC3000 *hrpL* under control of *PnptII*, Tc^r	Fouts et al. 2002
pCPP5042	pBluescriptII SK carrying 11.9-kb fragment from CUCPB5115, Ap^r	This study
pCPP5043	pBluescriptII SK carrying 4.1-kb *Bam*HI fragment from pCPP5042, Ap^r	This study
pCPP5044	pBluescriptII SK carrying *aadA1* from pHP45ΩSp/Sm^r, Ap^r Sp^r	This study
pCPP5045	pCPP5043 *ΔhopPtoA2::aadA1*, Ap^r Sp^r	This study
pCPP5046	pCPP54 carrying *Bam*HI fragment from pCPP5045, Tc^r Sp^r	This study
pCPP5047	pCPP45 carrying *Bam*HI fragment from pCPP5043, Tc^r	This study
pCPP5048	pBluescriptII SK carrying *hopPtoA2*, Ap^r	This study
pCPP5049	pFLAG-CTC carrying *hopPtoA2*, Ap^r	This study
pCPP5050	pJQ200SK carrying *hopPtoA1::uidA1::nptII* construct, Gm^r Km^r	This study
pCPP5051	pJQ200SK carrying *hopPtoA2::uidA1::nptII* construct, Gm^r Km^r	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin, Nx, nalidixic acid; Tc, tetracycline; Sp, spectinomycin; Sm, streptomycin, Rif, rifampicin.

Construction of the *hopPtoA1* marker exchange mutation in *P. syringae* pv. *tomato*.

A 4.2-kb *Mfe*I fragment containing *hrpW*, *hopPtoA1*, and part of ORF6 (Fig. 1A) was subcloned from pCPP3016 into the *Eco*RI site of pBluescriptII SK to make pCPP2437. pCPP2437 was digested with *Nar*I, the DNA overhanging ends were digested with Klenow, and the fragment was ligated to a *Sma*I fragment carrying a terminatorless *nptII* gene from pCPP2988 to construct pCPP2476. A *Bam*HI fragment containing the *nptII* cassette plus *P. syringae* pv. *tomato* DC3000 flanking DNA from pCPP2476 was inserted into pRK415 to construct pCPP2455. The mutated *hopPtoA1* was marker-exchanged into *P. syringae* pv. *tomato* DC3000 to construct strain CUCPB5106. The mutation was confirmed with DNA gel blot analysis. For complementation of *hopPtoA1*, a 3.0-kb *Sph*I fragment from pCPP2435 carrying *hopPtoA1* was ligated into the *Sph*I site of pUCP24 to construct pCPP2475, which should express *hopPtoA1* under the control of its native promoter.

Cloning of *hopPtoA2* and construction of its marker exchanged mutations in *P. syringae* pv. *tomato*.

Genomic DNA of the *P. syringae tomato* ΔCEL mutant CUCPB5115 was digested with *Hin*dIII; the 11.9-kb fragment carrying *hopPtoA2* was cloned into pBluescriptII SK to construct pCPP5042 and was sequenced. For gene replacement, pCPP5042 was digested with *Bam*HI and the 4.1-kb fragment containing *hopPtoA2* subcloned into pBluescriptII SK to obtain pCPP5043. The *aadA1* gene including its native promoter and no transcription termination signals was PCR amplified from pHP45ΩSp/Sm^r using primers p0809 and p0810. The amplified fragment was cloned into the *Hin*dIII sites of pBluescriptII SK to construct pCPP5044. pCPP5043 was digested with *Age*I and *Mlu*I and the 1.2-kb internal fragment from *hopPtoA2* was replaced with the terminatorless *aadA*1 from pCPP5044 to construct pCPP5045. The *Bam*HI fragment containing the *aadA1* cassette plus *P. syringae* pv.. *tomato* DC3000 flanking DNA from pCPP5045 was subcloned into pCPP54 to construct pCPP5046. The mutated *hopPtoA2* was marker-exchanged into *P. syringae* pv. *tomato* DC3000 and the *hopPtoA1* mutant strain CUCPB5106 to construct strains CUCPB5133 and CUCPB5134, respectively. The mutations were confirmed with DNA gel blot analyses. For complementation of *hopPtoA2*, the *Bam*HI fragment from pCPP5043 carrying *hopPtoA2* was ligated into the *Bam*HI site of pCPP45 to construct pCPP5047, which should express *hopPtoA2* under the control of its native promoter.

Construction of *hopPtoA1* and *hopPtoA2* fused to the FLAG epitope.

hopPtoA1 was amplified from pCPP3016 with Vent polymerase using primers p0408 and p0098 and ligated into the *Eco*RI and *Sal*I sites of pBluescriptII SK to construct pCPP3021. pCPP3021 was digested with *Nde*I and *Sal*I, and the fragment subcloned into pFLAG-CTC (Kodak, Rochester, NY, U.S.A.) to construct pCPP3020. *hopPtoA2* was PCR amplified from pCPP5042 with Vent polymerase using primers p0820 and p0821 and ligated into the *Hin*dIII and *Sal*I sites of pBluescriptII SK to construct pCPP5048. pCPP5048 was digested with *Nde*I and *Eco*RI, and the fragment subcloned into pFLAG-CTC (Kodak) to construct pCPP5049. pCPP3020 and pCPP5049 constructs produce HopPtoA1 and HopPtoA2 fused to the FLAG epitope under the control of the *tac* promoter.

Construction of genomic *uidA* transcriptional fusions of *hopPtoA1* and *hopPtoA2*.

To construct a transcriptional fusion of *hopPtoA1* with *uid*A, a 2.1-kb fragment containing the first 246 bp of the coding region of *hopPtoA1* and upstream sequences (Fig. 1A) was amplified from pCPP3016 with Deep Vent polymerase using primers p0885 and p0886. A 2.2-kb fragment including the last 477 bp of the *hopPtoA1* coding region and downstream sequence was amplified using primers p0887 and p0888. The digested fragments were ligated to an *uidA*::*nptII* cassette from pCPP2277 digested with *Xba*I and *Xho*I and to pJQ200SK digested with *Apa*I and *Sac*I to obtain pCPP5050. pCPP5050 was maker-exchanged into CUCPB5114 to obtain strain CUCPB5135. To construct *hopPtoA2* fused to *uid*A, a 2.2-kb fragment of sequence including the first 389 bp of the coding region of *hopPto*A2 and upstream sequences (Fig. 1B) was amplified from pCPP5042 with primers p0881 and p0882. A 1.7-kb fragment including the last 416 bp of the coding region of *hopPto*A2 and downstream sequence was amplified with primers p0883 and p0884. The digested fragments were ligated to *uidA-nptII* and pJQ200SK as indicated above to construct pCPP5051. pCPP5051 was maker-exchanged into CUCPB5114 to obtain strain CUCPB5136. CUCPB5135 and CUCPB5136 were transformed with pCPP5032, which expresses *hrpL* under the control of the *nptII* promoter, and with pCPP5031, which is the vector carrying only the *nptII* promoter.

Protein secretion assays.

To determine if HopPtoA1 and HopPtoA2 are secreted via the Hrp system, *E. coli* DH5α(pCPP2156) and *E. coli* DH5α(pCPP2368) were transformed with pCPP3020 and pCPP5049. pCPP2156 carries a functional type III secretion system from *Erwinia chrysanthemi*. pCPP2368 is a pCPP2156 derivative with a mini-*Tn5*Cm^r in the *hrpJ* operon (Ham et al. 1998). Cells were grown overnight on LM agar plus appropriate antibiotics at 37°C, scraped from the plates, washed twice with LM liquid medium, and resuspended in 40 ml LM plus antibiotics to an OD_{600} of 0.2. The cultures were grown at 30°C until the OD_{600} reached 0.4, then isopropyl-β-D-thiogalacto-

Table 3. Primers used in this study[a]

Primer	Sequence (5'-3')	Restriction enzymes
p0098	TCACAGTTGTCGACCGACCGCATAGG	*Sal*I
p0408	GAGGGAATTCATATGCACATCAACCGA	***Eco*RI** and *Nde*I
p0809	CGCCGCGAAGCTTACCGGTCTTGAACGAAT	***Hin*dIII** and *Age*I
p0810	GCTCGTAAGCTTGACGCGTCAGAAATGCCT	***Hin*dIII** and *Mlu*I
p0820	ACCGAAGCTTATTCATATGCACATCAACCA	***Hin*dIII** and *Nde*I
p0821	TGCAACCCACAGGCGAATTCCAAACG	*Eco*RI
p0881	GGCAGTGTATGGGGAGCTCTCAATCT	*Sac*I
p0882	CGAGGCAGCGGCATCTAGAGTGTTC	*Xba*I
p0883	GGTGCTCGAGCCGGGGTAAGTAAGT	*Xho*I
p0884	TTGACCTGGGCCCGGGCGTGACA	*Apa*I
p0885	CGATCAGCTCTAGAATCTGCGCATTGTGTA	*Xba*I
p0886	CCTTCTTTCACGAGCTCGAGGACTATCA	*Sac*I
p0887	CTGAGTGCGGGGCCCTCGTTGATAA	*Apa*I
p0888	AATGGGTGCTCGAGGAAGCCTGGTGT	*Xho*I

[a] Restriction sites are indicated with boldface and underline.

pyranoside was added to a final concentration of 500 μM, and incubation was continued until the OD_{600} was 0.8. The cell-bound and supernatant proteins were prepared as described by Ham and associates (1998), separated by polyacrylamide gel electrophoresis with 12% acrylamide and a Mighty Small II apparatus (Hoefer, San Francisco). Separated proteins were electrotransferred to Immobilon-P membranes (Millipore Co., Bedford, MA, U.S.A.) in a Hoefer transfer unit for 1 h at 80 mA at room temperature. Immunostaining with monoclonal M2 anti-FLAG antibody (Kodak) was performed using the Western-Light kit (Tropix, Bedford, MA, U.S.A.). Immunostaining with anti-β-galactosidase (Sigma, St. Louis) was also performed to detect the cytoplasmic control β-galactosidase. Membranes were exposed multiple times at time lengths ranging from 30 s to 20 min on Kodak OMAT X-ray films (Kodak) to record HopPtoA1, HopPtoA2, and β-galactosidase detection.

GUS assays.

CUCPB5114(pCPP5031), CUCPB5114(pCPP5032), CUCPB-5135(pCPP5031), CUCPB5135(pCPP5032), CUCPB5136-(pCPP5031) and CUCPB5136(pCPP5032) were grown in liquid AB-citrate (Chilton et al. 1974) at 30°C overnight. Cultures were diluted to an optical density of 0.3 and grown further for 6 h. Then 100 μl of liquid culture were used to determine GUS activity by the MUG (4-methylumbelliferyl β-D-glucuronic acid) method (Jefferson 1987) and a fluorometer Hoefer DyNA Quant 200 (Amersham Pharmacia Biotech Inc., San Francisco).

Plant assays.

Tobacco, tomato, and *Arabidopsis thaliana* plants were grown and inoculated with bacteria as described previously (Gopalan et al. 1996). For virulence assays, bacterial suspensions containing 10^4 CFU/ml were infiltrated into tomato leaves and monitored daily over a 4-day period for symptom development and bacterial multiplication. Plants were maintained under high humidity conditions in an incubator at 20°C and with a 12 h light and 12 h dark cycle.

To determine whether hopPtoA1 can elicit the HR in tobacco, inhibit the HR elicited by pHIR11, or complement the HR^- deficient phenotype of the pHIR11 *hopPsyA* mutant, a 3.0-kb *Sph*I fragment carrying the *hopPtoA1* operon was ligated into the *Sph*I site of a broad-host-range vector to construct pCPP2475, which should express *hopPtoA1* using its own promoter. *P. fluorescens* cells carrying a cosmid encoding the *P. syringae* pv. *syringae* 61 Hrp secretion system (pHIR11) or mutant derivatives of this cosmid (Alfano et al. 1996) was transformed with pCPP2475 and infiltrated into tobacco leaves.

DNA gel blots.

Total DNA (2 μg) was digested with restriction enzymes and separated by electrophoresis on 0.5% gels. DNA was transferred to Immobilon-N membrane (Millipore Co.) and blots were hybridized at 50°C for 8 h in HYB-9 DNA hybridization solution (Gentra Systems, Research Triangle Park, NC, U.S.A.) with a 1.5-kb *hopPtoA1* fragment that was amplified by PCR and then labeled with ^{32}P by using the Prime-It II kit (Stratagene). The membranes were washed four times in 1.0% sodium dodecyl sulfate (SDS) and 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) followed by two washes in 1.0% SDS and 0.2× SSC. Membranes were exposed to OMAT X-ray film (Kodak) for 4 to 12 h. Genomic DNA from *P. syringae* pathovars and other plant pathogenic bacteria was digested with *Eco*RI in experiments to determine if DNA sequences homologous to *hopPtoA1* were present in other plant pathogenic bacteria.

Microscopy.

P. syringae pv. *tomato* DC3000 wild-type, CUCPB5106, CUCPB5133, and CUCPB5134 strains were transformed with pGFP-TIR or pTB93F. Suspensions of each GFP-labeled strain at OD_{600} of 0.2 were prepared and diluted 1:20 with water. The diluted suspensions were infiltrated into *Arabidopsis* leaves with a blunt syringe, and the plants were incubated as indicated above for 40 hours. Ten fields per strain were observed with the 20× objective in a confocal laser-scanning microscope Bio-Rad MRC600 (Bio-Rad Laboratories). The average diameter of the colonies and their volumes (assuming a sphere) were calculated for every field observed. The frequency of colonies smaller than 250 μm^3 and the frequency of colonies equal to or larger than 250 μm^3 of each strain was entered in a contingency table and the observed cell counts compared by applying the chi-square test. The statistical procedures were applied using PROC FREQ with the options TABLE and CHISQ of SAS version 8.2 for Windows (SAS Institute Inc., Cary, NC, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI BLAST site: www.ncbi.nlm.nih.gov/BLAST/).

European Bioinformatics Institute, InterProScan sequence search page: www.ebi.ac.uk/interpro/scan.html.

Washington University in St. Louis, the Pfam database of protein domains and HMMs home page: pfam.wustl.edu/.

Imperial College of Science, Technology, and Medicine, 3D-pssm fold recognition server: www.sbg.bio.ic.ac.uk/~3dpssm.

National Center for Research Resources, Learning, observing and outputting protein patterns (Loopp) site: www.tc.cornell.edu/reports/NIH/resource/CompBiologyTools/loopp/.